

Lidocaine Inhibits Tyrosine Kinase Activity of the Epidermal Growth Factor Receptor and Suppresses Proliferation of Corneal Epithelial Cells

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Background: **Although lidocaine is recognized as an excellent topical corneal analgesic, its toxic effect on corneal epithelial cells limits its use during corneal epithelial wound healing. Mechanism of the impairment of corneal reepithelialization with lidocaine, however, has not been evaluated. The authors' previous study revealed that lidocaine inhibits the activity of tyrosine kinase receptors through the interaction with specific amino acid sequences around autophosphorylation sites, including acidic, basic, and aromatic amino acids. Epidermal growth factor receptor (EGFR), a tyrosine kinase receptor with an important role in epithelial cell proliferation after corneal wounding, also possesses these amino acids sequences around autophosphorylation sites. The authors hypothesized that lidocaine would suppress tyrosine kinase activity of EGFR and would impair corneal epithelial cell proliferation.**

Methods: **To investigate the effect of lidocaine (4 μM–40 mM) on epidermal growth factor (EGF)–stimulated autophosphorylation of EGFR, the authors studied purified EGFR in microtubes. They cultured human corneal epithelial cells (HCECs) with EGF and lidocaine to investigate the effect of lidocaine on cell proliferation and on autophosphorylation of EGFR in HCECs.**

Results: **Lidocaine (≥ 400 μM) significantly suppressed EGF-stimulated autophosphorylation of the purified EGFR. In the HCEC study, EGF alone stimulated cell proliferation and increased autophosphorylation of EGFR in HCECs. Lidocaine (≥ 400 μM) significantly suppressed both the proliferation of HCECs promoted by EGF and EGF-stimulated autophosphorylation of EGFR.**

Conclusion: **Lidocaine directly inhibits tyrosine kinase activity of EGFR and suppresses the corneal epithelial cell proliferation.**

LOCAL anesthetics can provide effective corneal anesthesia during corneal epithelial wound healing. Prolonged (or even a single) application of local anesthetics, however, causes delay of corneal reepithelialization after wounding.[1,2] Although topical lidocaine results in dose-dependent impairment of corneal epithelial wound healing,[3] the mechanism by which lidocaine negatively alters wound healing is not known.

Epidermal growth factor (EGF) is generally considered to be the main effector during corneal wound healing.[3–6] Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor present on the surface of most epithelial cells. Binding of EGF to the extracellular region of EGFRs leads to their dimerization and autophosphorylation of specific tyrosine residues of the intracellular activation region, and then results in activation of multiple downstream signaling pathways, leading to cell growth and proliferation.[7]

In the previous studies,[8–11] we showed that clinical concentration of lidocaine interacts with the aromatic ring of phenylalanine (F) and tyrosine (Y), with the negatively charged acidic amino acids, aspartic acid (D) and glutamic acid (E), and with the basic amino acids, lysine (K) and arginine (R). Lidocaine could bind around the sodium channel inactivation gate (*DIFMTEE* 1487–1493) followed by sodium channel block,[8,9] and also could bind around an autophosphorylation site of insulin receptor (*RDIY*^{1158}*ETDY*^{1162}*Y*^{1163}*R*) followed by the inhibition of its tyrosine kinase activity (table 1).[10,11] Inspection of the amino acid sequence of autophosphorylation sites of EGFR[12] showed that several autophosphorylation sites are the plausible binding sites for lidocaine (*EEKEY*^{845}HA*E*, *DADEY*^{992}LI, *RD*PH*Y*^{1105}Q*D*, *D*NP*DY*^{1148}QQ*DFF*, *E*NA*EY*^{1173}L*R*) (table 1). Therefore, we hypothesized that lidocaine interacts directly with the intracellular activation region of EGFR and then suppresses EGF signaling. To investigate the effect of lidocaine on autophosphorylation of EGFR, we used purified EGFR. We also studied changes in the proliferation of SV40-immortalized human corneal epithelial cells (HCECs)[13] by application of lidocaine into the cultured HCECs and evaluated the effect of lidocaine on tyrosine phosphorylation of EGFR in the HCECs.

Materials and Methods

Antiphosphotyrosine antibody (4G10) and anti-EGFR rabbit polyclonal antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). EGFR from human carcinoma A431 cells purified by affinity chromatography, and chemicals including lidocaine hydrochloride were from Sigma Chemical Co. (St. Louis, MO).

Human corneal epithelial cells were generously provided from Dr. Kaoru Sasaki (Department of Ophthalmology, Osaka University Medical School, Osaka, Japan)[13] and were maintained with Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% (vol/vol) fetal bovine serum, penicillin, streptomycin, choleratoxin, insu-

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Table 1. Hypothetical Binding Sites for Lidocaine in the Sodium Channel Inactivation Gate and Autophosphorylation Sites of Several Tyrosine Kinases

Receptors	Amino Acid Sequences												
Sodium channel	1485	G	Q	*D*	I	*F*	M	T	*E*	*E*	Q	*K*	1495
Insulin receptor	1154	T	*R*	*D*	I	*Y*	*E*	T	*D*	*Y*	*Y*	*R*	1164
IGFR	1127	T	*R*	*D*	I	*Y*	*E*	T	*D*	*Y*	*Y*	*R*	1137
EGFR	841	*E*	*E*	*K*	*E*	*Y*	H	A	*E*	G	G	*K*	851
	1101	*R*	*D*	P	H	*Y*	Q	*D*	P	H	S	*D*	1111
	1144	*D*	N	P	*D*	*Y*	Q	Q	*D*	*F*	*F*	P	1154
KGFR	766	T	N	*E*	*E*	*Y*	L	*D*	L	T	Q	P	776

Aromatic (F, Y), acidic (D, E), and basic (K, R) amino acids are italicized.

EGFR = epidermal growth factor receptor; IGFR = insulin-like growth factor receptor; KGFR = keratinocyte growth factor receptor.

lin, and EGF. The cells were grown in 25-cm^2 culture flasks, and the medium was changed every other day.

Autophosphorylation of Purified EGFR in the Presence of Lidocaine

Purified EGFR (1 μg protein) was phosphorylated with 0.2 mM adenosine triphosphate for 5 min at 37°C in a 50-μl incubation buffer (50 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). EGF (100 ng/ml), lidocaine (4 μM, 40 μM, 400 μM, 4 mM, 40 mM), or both were also added before incubation. In an *in vitro* study using purified EGFR, 100 ng/ml EGF was an appropriate concentration to stimulate EGFR.[14] After the incubation, the samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis by adding Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and boiling for 5 min, followed by Western blot analysis with antiphosphotyrosine antibody.

Cell Proliferation Assay

The cells were detached with a 0.25% trypsin/1 mM EDTA-4Na solution for 20 min. Subsequently, the cells were seeded onto 96-well plates (1×10^3 cells/well), and test compounds were added to the wells (100 μl in total for each well). Control medium contained Dulbecco's modified Eagle's medium, 1% (vol/vol) fetal bovine serum, penicillin, streptomycin, choleratoxin, and insulin. Test compounds consisted of the control medium and drugs of various concentrations (10 ng/ml EGF and 4 μM, 40 μM, 400 μM, 4 mM, and 40 mM lidocaine). Eight wells each were used for control and each drug concentration. Previous studies showed that 4–40 ng/ml EGF was appropriate to induce cell proliferation and tyrosine phosphorylation in a cell culture study using corneal epithelial cells.[15,16]

After incubation in a 37°C, 5% CO_2 environment for 5 days, the number of cells in each well was measured colorimetrically by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Briefly, 20 μl CellTiter 96® AQueous One Solution Reagent was added into each well. After 1 h of incubation, the absorbance of each well at 492 nm was recorded by a 96-well plate reader (Multiskan BICHROMATIC; Labsystems, Helsinki, Finland). After subtraction of the background absorbance (*i.e.*, the average absorbance of wells containing medium without any cells), the ratios of the absorbance can be interpreted as those of cell number of each well.

Analysis of the Effect of Lidocaine on Autophosphorylation of EGFR on Cultured HCEC

The cells were cultured on dishes to be confluent. Media were removed, and test compounds (10 ng/ml EGF; 4 μM, 40 μM, 400 μM, 4 mM, or 40 mM lidocaine; or both) were added (5 ml each). After incubation in a 37°C, 5% CO_2 environment for 5 min, the compounds were replaced with 5 ml phosphate-buffered saline. Each sample was scraped and harvested into the tubes. The supernatant was removed by centrifugation at 1,500 rpm for 5 min. Each sample was suspended in 0.6 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% [vol/vol] Nonidet P-40 [Nacalai tesque, Kyoto, Japan], 10% [vol/vol] glycerol, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 5 μg/ml aprotinin, 0.5 μg/ml pepstatin) and laid on ice for 30 min.

Insoluble material was removed by centrifugation at 15,000 rpm for 15 min. Aliquots of the supernatants containing equal amounts of protein, as determined using the Bradford protein assay with Bradford reagent (Sigma Chemical), were subjected to immunoprecipitation for 1 h at 4°C with anti-EGFR antibody. After the addition of protein A-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ), the immunoprecipitates were washed three times in a wash buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% [vol/vol] Nonidet P-40, 10% [vol/vol] glycerol, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 5 μg/ml aprotinin, 0.5 μg/ml pepstatin). The samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis by adding Laemmli sample buffer and boiling for 5 min.

The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% (vol/vol) acrylamide solving gels and transferred electrophoretically to nitrocellulose membrane (Bio-Rad Laboratories). The membranes were then blocked in 5% (wt/vol) dried milk in phosphate-buffered saline containing 0.1%

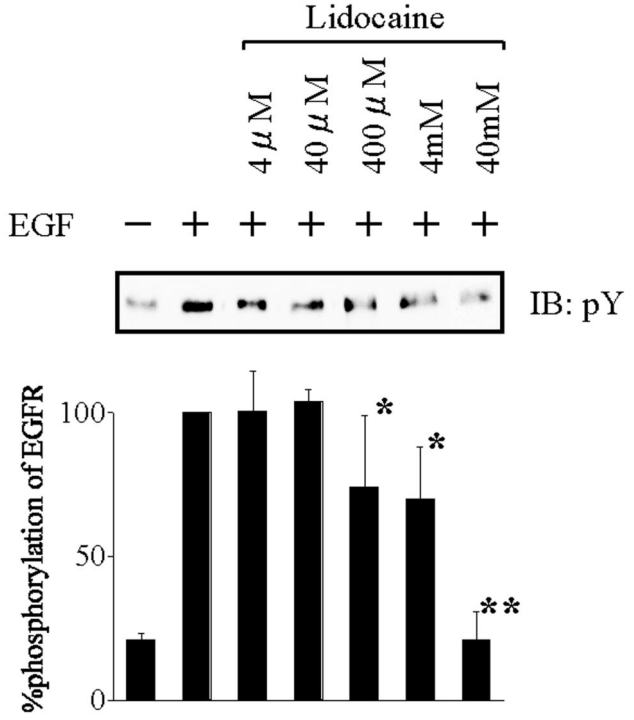

Fig. 1. Phosphorylation of purified epidermal growth factor receptor (EGFR) in the presence or absence of lidocaine. Purified EGFR was incubated in buffer containing 0.2 mM adenosine triphosphate with or without 100 ng/ml epidermal growth factor (EGF), lidocaine, or both for 5 min at 37°C. Lidocaine reduced EGF-stimulated tyrosine phosphorylation of EGFR. Result displayed in the *upper section* represents typical immunoblots (IB). pY = antiphosphotyrosine. * $P < 0.05$, ** $P < 0.01$ *versus* EGF-stimulated tyrosine phosphorylation without lidocaine; n = 4 for each lane.

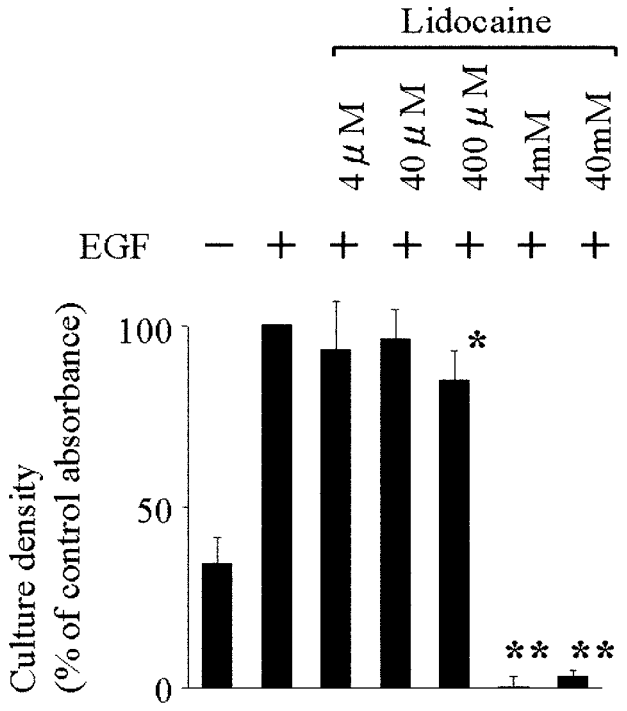

Fig. 2. Cell proliferation of human corneal epithelial cell in the presence or absence of lidocaine. The cells were incubated for 5 days with epidermal growth factor (EGF), lidocaine, or both. *Solid bars* represent basal and EGF-stimulated levels of culture density, which is a percent of control absorbance at EGF-stimulated level without lidocaine (100%). * $P < 0.05$, ** $P < 0.01$ *versus* EGF-stimulated level without lidocaine; n = 4 for each lane.

(vol/vol) polyoxymethylenesorbitan monolaurate (Tween 20; Sigma Chemical) for 1 h at room temperature and were then immunoblotted with appropriate antibody. The antigen antibody complexes were visualized by chemiluminescence luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA). Bands of interest were scanned and quantified by using LightCapture AE-6960 (ATTO Corporation, Tokyo, Japan).

Statistical Analysis

Data were analyzed by one-way analysis of variance with Bonferroni-corrected *post hoc* analysis. The statistical significance was established at the level of $P < 0.05$. All values are reported as mean ± SD.

Results

Lidocaine Suppresses EGF-stimulated Phosphorylation of Purified EGFR

Epidermal growth factor increased autophosphorylation of purified EGFR (fig. 1). EGF-stimulated responses of EGFR were taken as 100%. Unstimulated basal (absence of EGF) phosphorylation of EGFR was 20.8 ± 2.4% of the EGF-stimulated response. During EGF stimulation, lidocaine suppressed tyrosine phosphorylation of EGFR in a dose-dependent manner (73.8 ± 25.0, 69.7 ± 18.2, and 20.8 ± 10.1%, respectively, with 400 μM, 4 mM, and 40 mM lidocaine). These results suggest that lidocaine inhibited the EGF-stimulated tyrosine kinase activity of EGFR.

Lidocaine Suppresses Both Cell Proliferation of HCEC and Its Autophosphorylation of EGFR Stimulated by EGF

Figures 2 and 3 show the results of the HCEC study. EGF alone greatly stimulated HCEC proliferation, and EGF-stimulated responses of the proliferation were taken as 100% (fig. 2). Lidocaine (400 μM) significantly suppressed EGF-stimulated HCEC proliferation (84.9 ± 8.2%). In both 4 and 40 mM lidocaine, HCECs showed greatly reduced survival.

To assess tyrosine phosphorylation of EGFR in HCECs, equal amounts of protein from HCECs were subjected to immunoprecipitation with anti-EGFR antibody followed by immunoblotting with antiphosphotyrosine antibody or anti-EGFR antibody. EGF stimulation resulted in a marked increase in tyrosine phosphorylation of EGFR in HCECs. EGF-stimulated responses of EGFR were taken as 100% (fig. 3). Lidocaine (400 μM, 4 mM, and 40 mM), however, significantly attenuated EGF-stimulated tyrosine phosphorylation of EGFR (44.6 ± 26.1, 21.4 ± 12.9, and 21.7 ± 10.9%, respectively) relative to the control level (100%). EGFR protein levels did not differ among every lane.

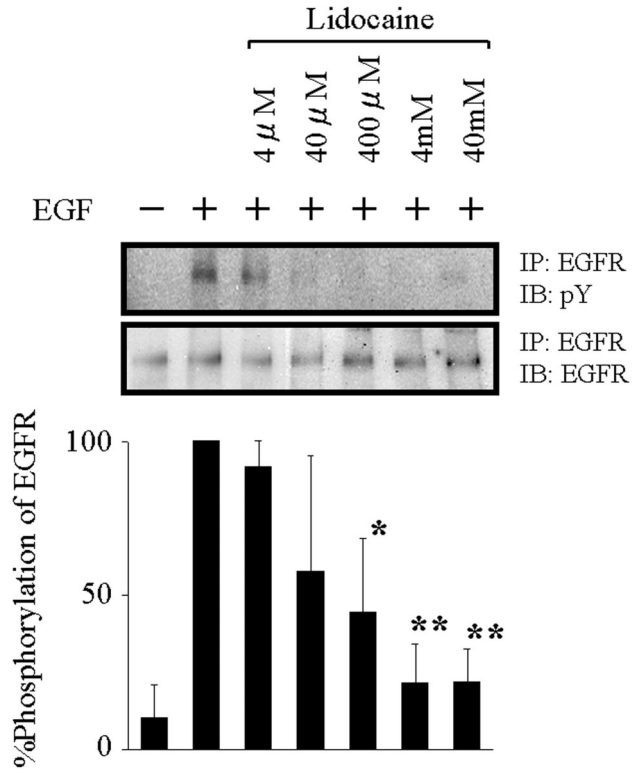

Fig. 3. Levels of tyrosine phosphorylation of epidermal growth factor receptor (EGFR) in the human corneal epithelial cell with or without lidocaine (4 μM–40 mM). The cells were incubated for 5 min with epidermal growth factor (EGF), lidocaine, or both. Equal amounts of human corneal epithelial cell protein were immunoprecipitated (IP) and immunoblotted (IB) with antiphosphotyrosine (pY) or anti-EGFR antibody. *Solid bars* represent basal and EGF-stimulated levels of tyrosine phosphorylation as a percent of EGF-stimulated phosphorylation without lidocaine (100%). EGF-stimulated tyrosine phosphorylation of EGFR (IP: EGFR; IB: pY) was attenuated by lidocaine (expressed as percent phosphorylation normalized to EGFR protein level). EGFR protein content (IP: EGFR; IB: EGFR) was not altered by the addition of lidocaine. Results displayed in the *upper panels* represent typical immunoblots of autophosphorylation of EGFR and immunoblots of protein content. * $P < 0.05$ and ** $P < 0.01$ *versus* EGF-stimulated tyrosine phosphorylation without lidocaine; n = 4 for each lane.

Discussion

Epidermal growth factor exists as a constant component of human tear fluid,[17] and both messenger RNA (mRNA) for EGF and mRNA for EGFR are present in HCECs.[18] EGF is a potent mitogen for corneal epithelial proliferation.[7] Therefore, the current study suggests that the attenuation of biologic functions of EGF by lidocaine is attributable to the inhibitory effect on the proliferation of HCECs.

Local anesthetic lidocaine is expected to interact with acidic, basic, and aromatic amino acids around several autophosphorylation sites in the EGFR by a variety combinations of noncovalent interactions, such as electrostatic, π-π stacking,[19] cation-π,[20] C-H-π,[21] and aromatic C-H···O hydrogen bonding[22] interactions. The π-π stacking provides interactions between the aromatic ring of lidocaine and aromatic amino acids (F and Y).[19] The aromatic ring of lidocaine can interact with the side chains of basic amino acids (K and R) through cation-π interaction.[20] Tertiary amine nitrogen of lidocaine interacts electrostatically with any of the negatively charged acidic amino acids (D and E). We observed that several autophosphorylation sites of tyrosine residues in EGFR are surrounded by acidic, basic, or aromatic amino acids. Moreover, lidocaine is an amphiphilic molecule and forms micelles. Therefore, it can be considered that lidocaine binds to tyrosine residues as a micelle by interacting with tyrosine itself and/or with acidic, basic, or aromatic amino acids by noncovalent interactions (table 1). Taking these facts together, we suggest that lidocaine inhibits EGF-stimulated tyrosine kinase activity of EGFR through the interaction with tyrosine residues themselves or with the residues residing around them in the autophosphorylation sites of the EGFR.

Kuroda *et al.*[8,9] studied interactions between local anesthetics and sodium channel inactivation gate–related peptides, and reported that local anesthetics, dibucaine and lidocaine, interact with the aromatic ring of phenylalanine (F1489) and with the negatively charged amino acids (D1487, E1492) around the sodium channel inactivation gate (DIFMTE 1487–1492). On the other hand, etidocaine is known to bind with aromatic amino acids, phenylalanine (F1764), and tyrosine (Y1771) in the IVS6 segment facing the pore of the sodium channel.[23] Therefore, in the previous study, we suggested that local anesthetics interact both with the inactivation gates and with the S6 segments in the sodium channel.[11] We found the close resemblance between the amino acid sequence around the sodium channel inactivation gate and that around the autophosphorylation site of insulin receptor (table 1)[10,11] and reported that lidocaine interacted with this site of insulin receptor.[10] Although there are no homologous alignments in hypothetical binding sites for lidocaine among EGFR, sodium channel, and insulin receptor in table 1, lidocaine would bind to autophosphorylation sites of EGFR, which are surrounded by acidic, basic, and aromatic amino acids, with noncovalent interactions. Other local anesthetics, which are amines having aromatic rings, can also interact with these hypothetical binding sites.

Various kinds of growth factors, such as EGF, keratinocyte growth factor, insulin-like growth factor, fibroblast growth factor, transforming growth factor, hepatocyte growth factor, and platelet-derived growth factor, play a key role in corneal wound healing.[6,7,24] Autophosphorylation sites of both keratinocyte growth factor and insulin-like growth factor receptors are surrounded by acidic, basic, or aromatic amino acids (*EEY*^{770}*LDL* in keratinocyte growth factor receptor, *RDIY*^{1131}*ETDY*^{1135}*Y*^{1136}*R* in insulin-like growth factor receptor).[25,26] Insulin, which was contained in our medium for the HCEC study, may also play a mitogenic role on corneal epithelial cells through insulin receptor,[27] and its autophosphorylation site is surrounded by these amino acids (*RDIY*^{1158}*ETDY*^{1162}*Y*^{1163}*R*).

We suggest that lidocaine interacts not only with EGFR but also with keratinocyte growth factor receptor and insulin-like growth factor receptor in addition to insulin receptor on the surface of HCECs (table 1). Moreover, the limitation of the current study is that we could not exclude the possible mechanisms of the inhibitory effect of lidocaine either upstream of the tyrosine phosphorylation sites of EGFR, such as the EGF/EGFR ligand binding site, or at downstream sites. Although autophosphorylation site of EGFR is an important target of lidocaine, other possible sites than EGFR would also play a role in the suppression of HCEC proliferation.

Clinical application of lidocaine, which ranges in concentration from 0.5 to 2% (approximately 20–80 mM), is associated with delay of corneal reepithelialization after corneal wounding.[1,2] Instillation of these concentrations of lidocaine, however, does not provide a measurable steady state concentration because it is diluted and washed away. To evaluate the effect of steady state lidocaine concentrations on corneal epithelial wound healing, Bisla and Tanelian[3] performed a tissue culture study using rabbit cornea with subepithelial wounds, and reported that continuous perfusion of 250 μg/ml (approximately 1 mM) lidocaine for 75 h delayed wound healing, but 100 μg/ml (approximately 400 μM) lidocaine did not. In the current study, 400 μM lidocaine suppressed HCEC proliferation slightly, but 40 μM lidocaine did not. We suggest that prolonged application of a low concentration of lidocaine (< 400 μM) can be used safely on the cornea.

High concentrations of lidocaine (4 and 40 mM) showed greatly reduced survival of HCECs in the current study. Tissue culture study using rabbit cornea also showed that 500 and 1,000 μg/ml (approximately 2 and 4 mM) lidocaine completely halted reepithelialization during corneal wound healing.[3] In cell culture studies, lidocaine (> 3 mM) induced apoptosis and necrosis in both dose-dependent and time-dependent manners.[28–30] Taken together, these results show that prolonged application of high concentrations of lidocaine induces cell death. The inhibitory effect of lidocaine on EGFR might not be a single cause of the halt in cell proliferation. Other mechanisms, which induce apoptosis or necrosis in cultured HCECs, might also be causes of the effect of high-concentration (4 and 40 mM) lidocaine on HCEC proliferation.

In conclusion, lidocaine directly inhibits tyrosine kinase activity of EGFR. This mechanism may be one of the causes of corneal toxicity of topical lidocaine after corneal wounding.

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